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Submitted on 12 Apr 2023

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Cryptococcus gattii Species Complex as an Opportunistic Pathogen: Underlying Medical Conditions Associated with the Infection

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ABSTRACT The Cryptococcus gattii species complex has often been referred to as a primary pathogen due to its high infection frequency among apparently immunocompetent patients. In order to scrutinize the immune status of patients and the lineages of etiologic agents, we analyzed patient histories and the molecular types of etiologic agents from 135 global C. gattii cases. Eighty-six of 135 patients had been diagnosed as immunocompetent, although some of them had underlying medical issues, and 49 were diagnosed as immunocompromised with risk factors similar to those seen in Cryptococcus neoformans infection. We focused on the 86 apparently immunocompetent patients and were able to obtain plasma from 32 (37%) to analyze for the presence of autoantibodies against the granulocyte-macrophage colony-stimulating factor (GM-CSF) since these antibodies have been reported as a hidden risk factor for C. gattii infection. Among the 32 patients, 25 were free from any known other health issues, and 7 had various medical conditions at the time of diagnosis for cryptococcosis. Importantly, plasma from 19 (76%) of 25 patients with no recognized underlying medical condition showed the presence of GM-CSF autoantibodies, supporting this antibody as a major hidden risk factor for C. gattii infection. These data indicate that seemingly immunocompetent people with C. gattii infection warrant detailed evaluation for unrecognized immunologic risks. There was no relationship between molecular type and underlying conditions of patients. Frequency of each molecular type was related to its geographic origin exemplified by the over-representation of VGIV in HIV-positive (HIV+) patients due to its prevalence in Africa.

IMPORTANCE The C. neoformans and C. gattii species complex causes cryptococcosis. The C. neoformans species complex is known as an opportunistic pathogen since it primarily infects immunocompromised patients. C. gattii species complex has been referred to as a primary pathogen due to its high infection frequency in apparently immunocompetent people. We analyzed 135 global cases of C. gattii infection with documented patient history. Eighty-six of 135 patients were originally diagnosed as immunocompetent, although some of them had underlying medical issues, and 49 were diagnosed as immunocompromised with risk factors similar to those seen in Cryptococcus neoformans infection. We focused on the 86 apparently immunocompetent patients and were able to obtain plasma from 32 (37%) to analyze for the presence of autoantibodies against the granulocyte-macrophage colony-stimulating factor (GM-CSF) since these antibodies have been reported as a hidden risk factor for C. gattii infection. Among the 32 patients, 25 were free from any known other health issues, and 7 had various medical conditions at the time of diagnosis for cryptococcosis. Importantly, plasma from 19 (76%) of 25 patients with no recognized underlying medical condition showed the presence of GM-CSF autoantibodies, supporting this antibody as a major hidden risk factor for C. gattii infection. These data indicate that seemingly immunocompetent people with C. gattii infection warrant detailed evaluation for unrecognized immunologic risks. There was no relationship between molecular type and underlying conditions of patients. Frequency of each molecular type was related to its geographic origin exemplified by the over-representation of VGIV in HIV-positive (HIV+) patients due to its prevalence in Africa.
immunocompetent and 49 as immunosuppressed with similar underlying conditions reported for *C. neoformans* infection. A significant number of *C. gattii* patients without known underlying conditions possessed autoantibodies against granulocytes-macrophage colony-stimulating factor (GM-CSF) in their plasma, supporting the presence of GM-CSF antibodies as a hidden risk factor for *C. gattii* infection. No relationship was found between *C. gattii* lineages and the underlying conditions except for overrepresentation of the molecular type VGIV among HIV+ patients due to the prevalence of VGIV in Africa.

**KEYWORDS** *Cryptococcus gattii* species complex, opportunistic pathogen, anti-GM-CSF autoantibodies, underlying medical conditions, molecular epidemiology

Although the two etiologic agents of cryptococcosis, *C. neoformans* and *C. gattii* species complexes, share 85 to 90% genomic identity (1), their global distribution and the patient populations affected are different. *C. neoformans* is the dominant cause of cryptococcosis. The majority of patients affected are those with immunosuppression (2–4), and hence, *C. neoformans* is considered an opportunistic pathogen. Globally, *C. gattii* has been a significantly less frequent cause of cryptococcosis, and clinical cases have been reported more often in patients with no recognized immunosuppression (2, 5, 6). Thus, *C. gattii* has been regarded as a primary pathogen (5, 7–9).

According to an analysis of 2,046 human clinical and veterinary cryptococcal isolates recovered from 48 countries, 80% belonged to the *C. neoformans* and only 20% to the *C. gattii* species complex, even though the geographic distributions of the two species complexes were similar (48% *C. neoformans* versus 52% *C. gattii*) in the environment of 21 countries (10). This suggests that environmental distribution fails to explain the disparity in clinical detection.

The *C. gattii* species complex contains four major lineages/molecular types, and recently, two more have been described (11, 12). The four major molecular types (VGI to VGIV) have been isolated from people with and without immunosuppression, but apparent host risks vary depending on the molecular type (6). The VGIII and VGIV lineages prevalent in tropical and subtropical regions most frequently are recognized as the cause of cryptococcosis in patients with HIV/AIDS (6, 13–16) and less frequently from immunocompetent patients (6). Infections with VGI and VGII lineages have been recognized relatively more frequently in people without clear medical risks (6, 17). If the VGI and VGII lineages are primary pathogens, why are there so few cases of travel-associated cryptococcosis reported among the tourists who have visited Vancouver, where a large *C. gattii* outbreak due to VGII lineage occurred from 1999 to 2007 (17)?

The annual number of tourists visiting British Columbia/Vancouver, a VGII-rich environment (18), is in the several millions (www.bcstats.gov.bc.ca). To date, only five cases of Vancouver travel-associated *C. gattii* infections have been documented in medical literature (19–23), and no case clusters have been reported, though tourists often travel in groups.

Differences in recognized infection despite widespread exposure raises questions about relative microbial pathogenicity and host risks (24, 25). The concept that previously healthy patients (diagnosed as immunocompetent) who suffered from *C. gattii* meningitis (CM) have certain hidden risk factors has emerged in 2014 (24, 25). Plasma from 6 of 8 Australian and 1 Chinese previously healthy *C. gattii* patients contained functional antibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF) (24). Since then, two reports on GM-CSF-neutralizing antibodies as a hidden risk factor for *C. gattii* infection in seemingly immunocompetent patients have followed (19, 26). It is understandable why GM-CSF-neutralizing antibodies predispose one to cryptococcosis since GM-CSF plays a crucial role in alveolar macrophage differentiation and development of normal innate immune function in the lung (27). Although anti-cytokine autoantibodies are increasingly recognized as important mechanisms of pathogenesis for various diseases (28), the prevalence of GM-CSF autoantibody among the healthy population is not...
known. It is, however, estimated to be 0.37 per 100,000 persons based on the rate of acquired pulmonary alveolar proteinosis (PAP) in which GM-CSF autoantibodies are the main cause (29).

What is not clear is the reason for more cases of *C. gattii* infection associated with these antibodies than with *C. neoformans* infection (24, 30, 31). Recently Perrineau et al. (31) and Panackal et al. (32) reported cases of central nervous system (CNS) infection caused by *C. neoformans* var. *grubii* in an HIV-seronegative patient with functional GM-CSF autoantibodies. This indicates that GM-CSF autoantibodies are more pronounced but not an exclusive risk factor for *C. gattii* infection. Since the routine diagnostic immunological profiling does not usually include the testing for levels of anti-cytokine autoantibodies, the presence of GM-CSF-neutralizing antibodies remains a hidden risk factor for cryptococcosis.

In this study, we analyzed microbial and host characteristics from 135 *C. gattii* infections representing global distribution to investigate the underlying medical conditions and determine the prevalence of GM-CSF autoantibodies among previously healthy people infected with *C. gattii*. We also assessed the relationship between underlying conditions and *C. gattii* lineages. The relationship between *C. gattii* molecular types and host risks was assessed further using data derived from the global cryptococcal database that incorporates multilocus sequencing type (MLST).

**RESULTS AND DISCUSSION**

**Molecular types of *C. gattii* isolates from patients with known clinical background.**

Our study included 135 *C. gattii* cases in which patients’ clinical history had been well documented (Table S1 in the supplemental material). They were from 5 continents, including 40 cases from Africa, 50 from North and South America, 12 from Asia, 7 from Europe, and 26 from Australia (Fig. 1A). Of these, 132 had central nervous system (CNS) infection, 2 isolated pneumonia, and 1 had a spinal epidural abscess. Of the original 135 isolates, 2 isolates from the patients SCA-2 (26) and LDS-107 (this study) had been discarded after being identified to the species level, and only 133 were available for molecular typing. We analyzed molecular types of 124 isolates in this study using the *URA5* restriction fragment length polymorphism (RFLP) and matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), and those of the remaining 9 isolates had been reported previously (24). Eighty-six (63.7%) of the 135 patients were originally considered to be lacking underlying risks (Fig. 1B to F; Table S1), and 49 (36.3%) patients were diagnosed as having recognized risks for cryptococcal infection, including HIV/AIDS, corticosteroid receipt, diabetes mellitus, cancer, or other immunosuppression. The 135 isolates represented all four major *C. gattii* lineages, which include 62 VGI (45.9%), 29 VGII (21.5%), 19 VGIII (14.1%), and 23 VGIV (17.0%), and 2 molecular type unknown (1.5%) (Fig. 2A). The molecular type determined by *URA5* RFLP analysis and MALDI-TOF MS score agreed for all isolates tested except for one (99.2% agreement), PW-039 (Table S2; Fig. S1). The distribution of each molecular type in different geographic regions agreed with previous findings in that the VGI is not only the most frequent but is especially common in Asia, Europe, and Australia, while VGII and VGIII are most prevalent in North and South America (6, 33). The isolates of VGIV we collected were all from Africa, where VGIV is known to be the most common molecular type of *C. gattii* among clinical isolates, especially from people with HIV/AIDS (6, 15, 33, 34).

The 86 isolates from patients considered to be immunocompetent included 43 VGI (69.4% of total cases due to VGI), 22 VGII (75.9% of total cases due to VGII), and 19 VGIII (100% of total cases due to VGIII) (Fig. 3); this included no VGIV and 2 isolates of molecular type unknown. Although the distribution of molecular type across patients with and without immunosuppression is similar to the previous report by Chen et al. (6), our isolates from the patients considered immunocompetent were lower in VGI and VGII (69.4% and 75.9% versus 92.8% and 88.9%) while higher in VGIII (100% versus 75.8%) than the previous reports (6, 35) (Fig. 3). The total number of VGI isolates included in the previous report (6) was 77 from immunocompetent and 6 from immunocompromised patients, and VGII
The prevalence of GM-CSF autoantibodies in the plasma of 32 C. gattii-infected patients. Plasma was available for detection of GM-CSF autoantibodies only from 32 of 135 C. gattii patients, which included 3 patients with known immunosuppressive
conditions, such as lymphoma and receipt of corticosteroid, and 29 previously healthy with no recognized immunosuppression (Table 1; Fig. 2B). Four of the 29 previously healthy patients considered immunocompetent had other medical issues, including menometrorrhagia, increased regulatory T cells (Treg), and cerebrospinal eosinophilia, and 25 had no known comorbid conditions at the time of diagnosis for cryptococcosis. Although considered immunocompetent, 20 (69%) of those 29 patients were positive for neutralizing antibodies against the GM-CSF (Table 1; Fig. 4A and B), indicating certain immune system abnormality. Except for the patient with menometrorrhagia, all patients with other medical issues before the onset of cryptococcosis, such as increased Treg and CSF eosinophilia or history of corticosteroid therapy, were negative for GM-CSF autoantibodies. This indicates that 19 (76%) of the 25 C. gattii patients without any known other medical issue had GM-CSF autoantibody as a hidden risk factor (Table 1; Fig. 4A). Our observation also raises the question of whether increased Treg or CSF eosinophilia played any role in the patient’s susceptibility to C. gattii or they were the by-product of C. gattii infection. Corticosteroid therapy is a known risk

FIG 2 Molecular types of isolates obtained from patients. (A) Molecular types of total 135 clinical isolates. (B) Molecular types of 32 isolates from patients whose plasma was tested for the presence of functional GM-CSF autoantibodies. (C) Molecular types of 20 isolates from patients with anti-GM-CSF autoantibodies.

FIG 3 Molecular types of the total 133 isolates obtained from patients considered to be immunocompetent or immunosuppressed at the time of diagnosis for cryptococcosis.
for cryptococcosis (2, 37), although it is poorly understood whether short-term therapy for skin lesions, which does not result in clear immunosuppression, could predispose one to cryptococcosis. Figure 2C and Table 1 show that 9 of the 20 GM-CSF antibody-positive patients were infected by VGI (45%); the remaining 11 (55%) patients were infected by VGII (4, 20%), VGIII (5, 25%), and 2 (10%) by isolates of unknown molecular type. Frequency of the three molecular types, VGI to VGIII, among the cases with antibody-positive plasma reflected the geographic origin of the patients; nearly 67% of the antibody-positive VGI patients were from Australia/Asia, where VGI is the most prevalent type, and 33% were from North America (Table 1). Antibody-positive patients infected by VGII and VGIII were all from North America where VGII and VGIII are most common, except for one VGII from Australia (6, 33). The lack of VGIV among the GM-CSF antibody-positive patients is expected since the patients’ plasma for the antibody test all came from the geographic regions where cryptococcosis due to VGIV is rarely reported (6, 33). Our data clearly suggest that the presence of GM-CSF-neutralizing antibody is a hidden risk for C. gattii infection in otherwise healthy individuals and suggest that the antibody test should be included in the immunologic profiling when previously healthy patients present with C. gattii infections. It is important to detect the presence of anti-GM-CSF autoantibody since it could lead to pulmonary alveolar proteinosis (PAP), a chronic lung disease characterized by abnormalities of surfactant

<table>
<thead>
<tr>
<th>Patient/isolate</th>
<th>Region</th>
<th>Source of isolate</th>
<th>Molecular type</th>
<th>Underlying issues at diagnosis</th>
<th>Ab‡</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>Australia (NSW)</td>
<td>CSF</td>
<td>VGI</td>
<td>None</td>
<td>+</td>
<td>24</td>
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<tr>
<td>A2</td>
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<td>CSF</td>
<td>VGI</td>
<td>None</td>
<td>+</td>
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</tr>
<tr>
<td>A3</td>
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<td>VGI</td>
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<td>+</td>
<td>24</td>
</tr>
<tr>
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<td>CSF</td>
<td>VGI</td>
<td>None</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>A5</td>
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<td>VGI</td>
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<td>+</td>
<td>24</td>
</tr>
<tr>
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<td>24</td>
</tr>
<tr>
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</tr>
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<td>VGII</td>
<td>None</td>
<td>-</td>
<td>24</td>
</tr>
<tr>
<td>CZ18</td>
<td>China (Shanghai)</td>
<td>CSF</td>
<td>VGI</td>
<td>None</td>
<td>+</td>
<td>24</td>
</tr>
<tr>
<td>SE744</td>
<td>USA (N. Carolina)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>+</td>
<td>19</td>
</tr>
<tr>
<td>SCA-1</td>
<td>USA (California)</td>
<td>Spinal abscess</td>
<td>VGIII</td>
<td>None</td>
<td>+</td>
<td>26</td>
</tr>
<tr>
<td>SCA-2</td>
<td>USA (California)</td>
<td>Brain specimen</td>
<td>? ‡</td>
<td>None</td>
<td>+</td>
<td>26</td>
</tr>
<tr>
<td>NIH-34</td>
<td>USA (New Jersey)</td>
<td>CSF</td>
<td>VGIII</td>
<td>None</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>NIH-435</td>
<td>USA (New Jersey)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>NIH-653</td>
<td>USA (Maryland)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>NIH-754</td>
<td>USA (Washington DC)</td>
<td>CSF</td>
<td>VGIII</td>
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<td>+</td>
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</tr>
<tr>
<td>R54</td>
<td>USA (Alabama)</td>
<td>CSF</td>
<td>VGII</td>
<td>Corticosteroid Rx c</td>
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</tr>
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<td>R56</td>
<td>USA (S, Carolina)</td>
<td>Pleural fluid</td>
<td>VGI</td>
<td>Corticosteroid Rx c</td>
<td>-</td>
<td>This study</td>
</tr>
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<td>BAL fluid/lung</td>
<td>VGII</td>
<td>Menometrorrhagia</td>
<td>+</td>
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<td>CSF</td>
<td>VGII</td>
<td>Lymphoma</td>
<td>-</td>
<td>This study</td>
</tr>
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<td>USA (Oregon)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>LDS-20</td>
<td>Canada (Vancouver)</td>
<td>BAL fluid</td>
<td>VGII</td>
<td>Increased Tregs ‡ d</td>
<td>-</td>
<td>This study</td>
</tr>
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<td>LDS-28</td>
<td>USA (Washington St.)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>LDS-36</td>
<td>USA (California)</td>
<td>CSF</td>
<td>VGIII</td>
<td>CSF eosinophilia ‡ d</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>LDS-49</td>
<td>USA (California)</td>
<td>CSF</td>
<td>VGIII</td>
<td>CSF eosinophilia ‡ d</td>
<td>-</td>
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<td>LDS-58</td>
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<td>LDS-64</td>
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<td>CSF</td>
<td>VGII</td>
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<td>-</td>
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<tr>
<td>LDS-76</td>
<td>Canada (Vancouver)</td>
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<td>+</td>
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<td>LDS-96</td>
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<td>CSF</td>
<td>VGIII</td>
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<td>+</td>
<td>This study</td>
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<tr>
<td>PCG001</td>
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<td>VGII</td>
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<td>-</td>
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<tr>
<td>DW-1</td>
<td>USA (California)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>LDS-107</td>
<td>USA (California)</td>
<td>CSF</td>
<td>VGII</td>
<td>None</td>
<td>+</td>
<td>This study</td>
</tr>
<tr>
<td>Total (n = 32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>Positive</td>
</tr>
</tbody>
</table>

‡ NSW, New South Wales, Ab, anti-GM-CSF autoantibody.
* Isolates not available for molecular typing.
+ Patients used corticosteroid for skin condition 3 months before C. gattii infection.
- Conditions found at the time of cryptococcosis diagnosis.
+ VGI, n = 14; VGII, n = 9; VGIII, n = 7; molecular type unknown, n = 2.

for cryptococcosis (2, 37), although it is poorly understood whether short-term therapy for skin lesions, which does not result in clear immunosuppression, could predispose one to cryptococcosis. Figure 2C and Table 1 show that 9 of the 20 GM-CSF antibody-positive patients were infected by VGI (45%); the remaining 11 (55%) patients were infected by VGII (4, 20%), VGIII (5, 25%), and 2 (10%) by isolates of unknown molecular type. Frequency of the three molecular types, VGI to VGIII, among the cases with antibody-positive plasma reflected the geographic origin of the patients; nearly 67% of the antibody-positive VGI patients were from Australia/Asia, where VGI is the most prevalent type, and 33% were from North America (Table 1). Antibody-positive patients infected by VGII and VGIII were all from North America where VGII and VGIII are most common, except for one VGII from Australia (6, 33). The lack of VGIV among the GM-CSF antibody-positive patients is expected since the patients’ plasma for the antibody test all came from the geographic regions where cryptococcosis due to VGIV is rarely reported (6, 33). Our data clearly suggest that the presence of GM-CSF-neutralizing antibody is a hidden risk for C. gattii infection in otherwise healthy individuals and suggest that the antibody test should be included in the immunologic profiling when previously healthy patients present with C. gattii infections. It is important to detect the presence of anti-GM-CSF autoantibody since it could lead to pulmonary alveolar proteinosis (PAP), a chronic lung disease characterized by abnormalities of surfactant
metabolism. PAP impairs the function of alveolar macrophages and can cause respiratory insufficiency (29). It is also noteworthy that one patient considered immunocompetent and lacking GM-CSF antibodies had a splice-site mutation in STAT3 when the whole exon of his genome was sequenced (data not shown). This suggests that exon sequencing may reveal other hidden risk factors in those seemingly immunocompetent patients infected by *C. gattii*.

The relationship between underlying conditions of known immunosuppression and molecular types of the *C. gattii* species complex. Of the 135 patients, 49 (36.3%) had known immunosuppression. The major immunosuppression identified among patients infected by the molecular types VGI, VGII, VGIII, and VGIV were similar to those associated with *C. neoformans* infection, including HIV infection, cancer, corticosteroid therapy, and other immunosuppressive conditions (Table S1). There was a clear relationship between geographic origin of patients and the prevalence of *C. gattii* molecular type, but no clear relationship was found between *C. gattii* molecular type and the immunosuppressive conditions except that all VGIV isolates we studied were from HIV-associated cryptococcosis. The predominance of VGIV among the HIV-associated *C. gattii* cases was expected since nearly 90% of the HIV-associated *C. gattii* isolates we have collected were from South Africa and Botswana, where VGIV is the predominant molecular type found in AIDS patients (10, 15, 33). A strong association of VGIV with AIDS patients is supported by the fact that we found no VGIV isolates in our stock cultures from the pre-HIV era (38). The VGIV isolates are serotype C and are found mostly in tropical and subtropical regions, such as India, Sub-Saharan Africa, Mexico, and Columbia, but infrequently in temperate zones (10). Finding no VGIV infection among our 86 patients originally diagnosed as immunocompetent (Fig. 3) supports the fact that VGIV most commonly affects HIV+ patients more than any other type of immunosuppressed patients.

The prevalence of VGI (54.5%, 12/22 cases) among the isolates from South African HIV-associated *C. gattii* infection, however, was significantly higher than expected.
Previous surveys record no VGI infection (39) or only 27% of all C. gattii infections in South Africa (10, 40). All 13 isolates from HIV-associated C. gattii cases in Botswana were VGIV, and VGI was lacking even though the country borders with South Africa. Only three VGII and no VGIII isolates were found among the 40 isolates from C. gattii infections in Africa. This is not surprising since VGII is infrequently found in Africa (6, 33, 39, 40), and VGIII is rarely found outside the American continent (33, 35, 41, 42).

**Underlying conditions of 179 patients infected by C. gattii species complex recorded in the global MLST database.** Of the 179 isolates from patients with known clinical history in the global MLST database, 47.5% (85/179) were from patients diagnosed as immunocompetent, and 52.5% (94/179) of the isolates were from those with immunosuppressive underlying conditions (Table 2). The frequency of C. gattii species complex recovered from patients recognized as immunocompetent among the 179 (https://mlst.mycologylab.org) (Table 2) was significantly lower ($P < 0.0001$) than in the previous report (6) or the present study. The disparity between the previous data and the MLST database on the frequency of the two types of patients is basically due to the differences in the frequency of the two groups infected by VGI and VGII lineages, but not those infected by VGIII and VGIV. The frequency of immunocompetent versus immunosuppressed patients infected by VGII and VGI was not significantly different ($P = 0.0963$ and $0.3693$, respectively) between the two data sets, although the sample size was too small to be statistically significant.

As Table 2 shows, the most common underlying immunosuppression in patients infected by the C. gattii species complex was HIV infection, regardless of the molecular types of the etiologic agent. Although the number of patients infected by VGII and VGI in the MLST database with clinical information is too small (15 and 12, respectively), diabetes was commonly identified as an underlying condition in infection by all four lineages. Underlying conditions, such as systemic lupus erythematosus (SLE), were found among patients infected by VGI and VGII. Other underlying conditions, such as chronic myelogenous leukemia (CML), CD4 lymphopenia, and cirrhosis, were found

### Table 2: Available metadata from MLST database on C. gattii

<table>
<thead>
<tr>
<th>Molecular type</th>
<th>No. of isolates in DB</th>
<th>No. of isolates from known clinical background</th>
<th>No. (%) immunocompetent (previously healthy)</th>
<th>No. (%) immunocompromised HIV+</th>
<th>Other</th>
<th>Underlying issues other than HIV (no. of patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGI</td>
<td>354</td>
<td>40</td>
<td>20 (50)</td>
<td>11 (27.5)</td>
<td>9 (22.5)</td>
<td>Cirrhosis (1), SLE (3), tumor-like structure (1), pregnancy (1), CML (1), diabetes (1), CD4 lymphopenia (1)</td>
</tr>
<tr>
<td>VGII</td>
<td>862</td>
<td>112</td>
<td>55 (49.1)</td>
<td>52 (46.4)</td>
<td>5 (4.5)</td>
<td>SLE (1), diabetes (2), alcoholic (1), MLS (1) Hepatoma (1), diabetes (1), depression (1), hypertension (1), paracoccidioidomycosis (1), strongyloidiasis (1), malnutrition (1), immunosuppressed (4)</td>
</tr>
<tr>
<td>VGIII</td>
<td>210</td>
<td>15</td>
<td>7 (46.6)</td>
<td>1 (6.6)</td>
<td>5 (26.6)</td>
<td>Diabetes (1)</td>
</tr>
<tr>
<td>VGIV</td>
<td>28</td>
<td>12</td>
<td>3 (25)</td>
<td>8 (66.6)</td>
<td>1 (8.3)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1454</td>
<td>179</td>
<td>85 (47.4)</td>
<td>72 (40.2)</td>
<td>22 (12.2)</td>
<td></td>
</tr>
</tbody>
</table>

DB, database; SLE, systemic lupus erythematosus; CML, chronic myelogenous leukemia; MLS, microphthalmia syndrome.
among VGI patients. These data indicate that the underlying immunosuppressive conditions which predispose patients to infections by the *C. gattii* and *C. neoformans* species complex overlap and there is no unique condition that predisposes patients only to the *C. gattii* species complex.

The important finding in the present study, however, is that the seemingly immunocompetent people with anti-GMCSF autoantibodies in their plasma are more often infected by *C. gattii* species complex than *C. neoformans* species complex.

**Conclusion.** It is important to recognize the *C. gattii* species complex as an opportunistic pathogen. Since GM-CSF-neutralizing antibodies are one of the most common hidden underlying conditions in previously healthy *C. gattii* patients, the antibody test should be implemented in *C. gattii* patients with no known immunosuppression. This test is especially important for patient management because patients with GM-CSF antibody may eventually develop further complications, such as pulmonary alveolar proteinosis (30).

The *C. gattii* patients considered immunocompetent and negative for the GM-CSF antibodies could carry other hidden risk factors, which may be revealed by whole-exon sequencing of the patient’s genome.

The known immunosuppressive underlying conditions that predispose patients to *C. gattii* infection are no different from those to *C. neoformans* infection except that the presence of anti-GM-CSF autoantibodies in plasma may pose more risk for infection by *C. gattii* species complex than the *C. neoformans* species complex.

**MATERIALS AND METHODS**

**Clinical data and samples.** Clinical data from 135 global cases of *C. gattii* infection were retrieved from studies approved by local institutional review boards. Isolates (*n* = 124) recovered from cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BAL fluid/lung tissue, or extrapulmonary sources were available for determination/confirmation of molecular types in our laboratory. Isolates were streaked on YPD agar (glucose 2%, yeast extract 1%, and peptone 2%) to test for their purity. Purified isolates were stored in 25% glycerol at −80°C until use. The species identity of each strain was confirmed by culturing on CGB media (43) in addition to testing for urease production (44), melanin production (45), and growth at 37°C.

Clinical data correlating with infections characterized to MLST type was obtained from the cryptococcal database (https://mlst.mycologylab.org). Clinical data were available for 179 of a total of 1,454 patients whose isolates had been characterized by MLST (Table 2).

**Identification of molecular types.** Molecular types (VGI to VGV) of the isolates were determined via *URA5* gene restriction fragment length polymorphism (RFLP) analysis (34) and MALDI-TOF MS (46). DNA from each culture was isolated (47), and the *URA5* gene was amplified using two PCR primers, *URA5* (5′-ATGTCCTCCCAAGCCCTCGACTCCG-3′) and SI01 (5′-TTAAGACCTCTGAAACCCGTACTCTC-3′). The PCR products were double digested with Sau96I and HhaI and were separated by 3% agarose gel electrophoresis (12) to compare with the *URA5* RFLP patterns of WM179 (VGI), WM178 (VGI), WM161 (VGI), WM779 (VGV). For MALDI-TOF MS analysis, MALDI target plates of the isolates were prepared as previously described (46). MALDI-TOF MS was performed on the MicroFlex LT mass spectrometer and analyzed by Biotyper 3.1 software (Bruker Daltonics, Inc.). Spectra were acquired over a mass/charge (m/z) ratio of 2,000 to 20,000. Spots were measured using 250 laser shots at 60 Hz in groups of 50 shots per sampling area. Both the Bruker and previously developed NIH custom databases included 22 *C. gatti* strains representing VGI to VGV. The best of two scores was recorded, and a cutoff of ≥1.80 was considered acceptable.

**Evaluation of anti-GM-CSF autoantibodies in patient’s plasma.** Frozen plasma was available from 32 people with *C. gattii* infection: 29 people had no known immunosuppression, and 3 had lymphoma (*n* = 1) and/or had received corticosteroids (*n* = 2). Among the 32, 12 cases have already been reported (Table 1), and 20 cases were from the present study. The presence of anti-GM-CSF autoantibodies in plasma samples was tested using previously described particle-based methodology (24, 30). Briefly, fluorescent magnetic beads (Bio-Rad) were covalently coupled to 2.5 μg of human GM-CSF (R&D Systems). Beads were transferred into magnet 96-well plate and incubated for 30 min with diluted plasma (1:100) on microplate shaker, washed, and incubated with streptavidin-phycocerythrin (Bio-Rad)-labeled goat anti-human IgG (eBioscience) for an additional 30 min. The plate was then run on Bio-Plex (Bio-Rad) instrument. Normal plasma and GM-CSF autoantibody-positive plasma were used as negative and positive controls, respectively. The antibodies’ neutralizing functionality was evaluated by flow cytometry to detect the phosphorylation of STAT5 in normal peripheral blood monocytes (PBMCs) after stimulation with GM-CSF in presence of the patient’s plasma as described (24, 30).

**Statistics.** Fisher’s exact test (Prism 8; GraphPad Software, San Diego, CA) was used to determine if there is a significant difference between the previous data and our data for the frequency of each molecular type from patients with immunosuppression versus those with no known immunosuppression. A *P* value of <0.05 was considered significant.
SUPPLEMENTAL MATERIAL
Supplemental material is available online only.

FIG S1, TIF file, 9.9 MB.
TABLE S1, DOCX file, 0.03 MB.
TABLE S2, DOCX file, 0.03 MB.

ACKNOWLEDGMENTS
This work was supported by the Division of Intramural Research (DIR), NIAID, NIH.
Public Health Service Grants (A173896 and A193257) supported the collection of isolates from Botswana by J.R.P.

REFERENCES
Cryptococcus gattii Is an Opportunistic Pathogen


